

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

To:

LICATA, Jane, Massey
Licata & Tyrrell P.C.
66 E. Main Street
Marlton, NJ 08053
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 27 March 2001 (27.03.01)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference RTSP-0047	
International application No. PCT/US00/00633	International filing date (day/month/year) 11 January 2000 (11.01.00)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address

LICATA, Jane, Massey
Law Offices of Jane Massey Licata
66 E. Main Street
Marlton, NJ 08053
United States of America

State of Nationality

State of Residence

Telephone No.

(609)810-1515

Facsimile No.

(609)810-1454

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☒ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address

LICATA, Jane, Massey
Licata & Tyrrell P.C.
66 E. Main Street
Marlton, NJ 08053
United States of America

State of Nationality

State of Residence

Telephone No.

856-810-1515

Facsimile No.

856-810-1454

Teleprinter No.

3. Further observations, if necessary:

The indication of a new company's name of the agent on the Demand (Form PCT/IPEA/401) has been considered a request for recording a change under Rule 92bis. In case of disagreement, the International Bureau should be notified immediately.

4. A copy of this notification has been sent to:

<input checked="" type="checkbox"/> the receiving Office	<input type="checkbox"/> the designated Offices concerned
<input type="checkbox"/> the International Searching Authority	<input checked="" type="checkbox"/> the elected Offices concerned
<input checked="" type="checkbox"/> the International Preliminary Examining Authority	<input type="checkbox"/> other:

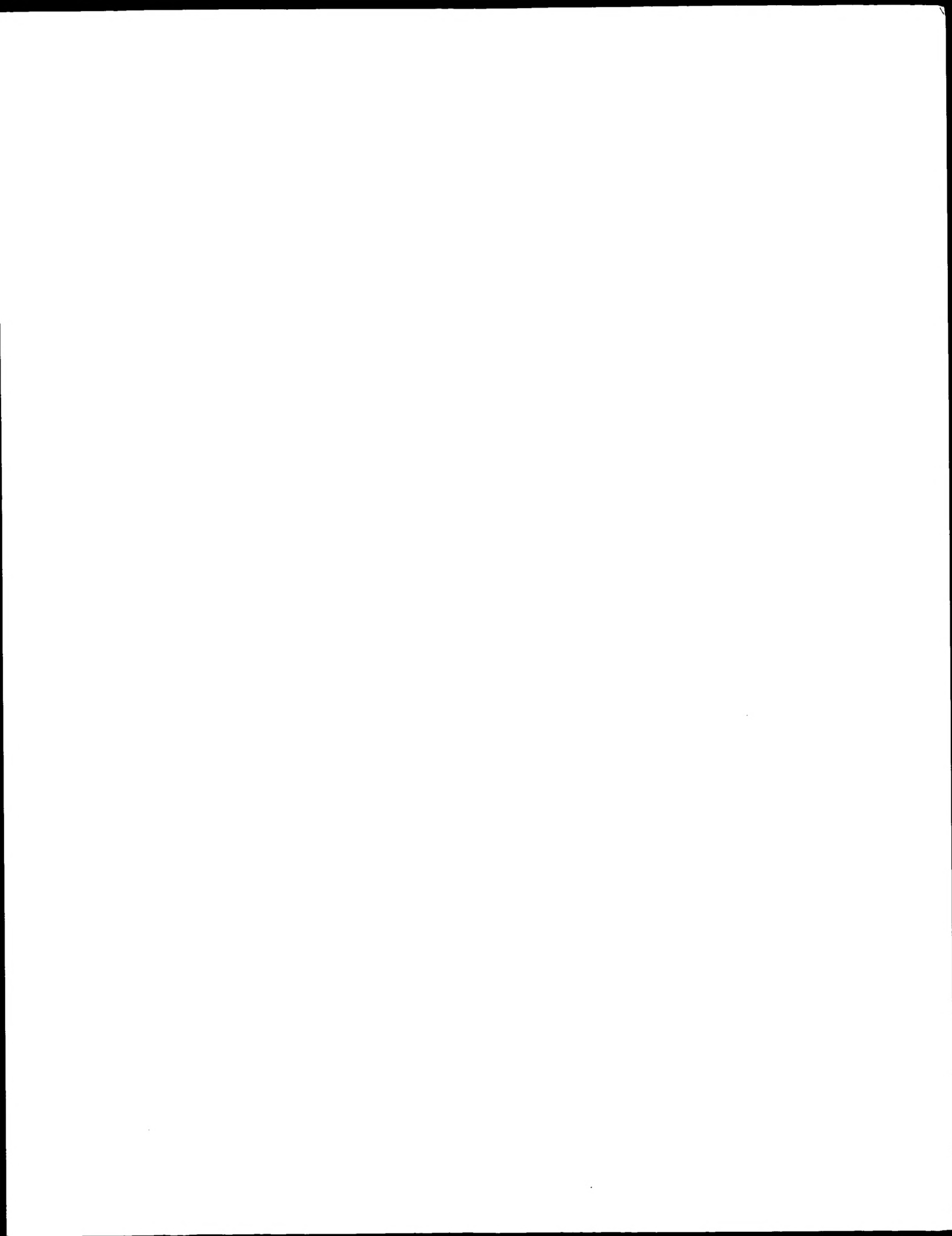
The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

Claudio Borton

Facsimile No.: (41-22) 740.14.35

Telephone No.: (41-22) 338.83.38



PATENT COOPERATION TREATY

PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

From the INTERNATIONAL BUREAU

To:

LICATA, Jane, Massey
Law Offices of Jane Massey Licata
66 E. Main Street
Marlton, NJ 08053
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 03 April 2000 (03.04.00)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference RTSP-0047	
International application No. PCT/US00/00633	
International publication date (day/month/year) Not yet published	
International filing date (day/month/year) 11 January 2000 (11.01.00)	Priority date (day/month/year) 25 June 1999 (25.06.99)
Applicant ISIS PHARMACEUTICALS, INC. et al	

- The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
- This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
- An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed to Rule 17.1(c)** which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
- The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed to Rule 17.1(c)** which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
25 June 1999 (25.06.99)	09/344,520	US	17 Marc 2000 (17.03.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: JANE MASSEY LICATA
LICATA & TYRELL P.C.
66 E. MAIN STREET
MARLTON, NJ 08053

Docket Sys. ☒
Status Rep. ☒
Docket Bond ☒

NP = 12.25.01

PCT

JUN 22 2001

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing
(day/month/year)

20 JUN 2001

Applicant's or agent's file reference
RTSP-0047

IMPORTANT NOTIFICATION

International application No.

PCT/US00/00633

International filing date (day/month/year)

11 JANUARY 2000

Priority Date (day/month/year)

25 JUNE 1999

Applicant

ISIS PHARMACEUTICALS, INC.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ANDREW WANG

Telephone No. (703) 308-0196

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT


(PCT Article 36 and Rule 70)

Applicant's or agent's file reference RTSP-0047	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/00633	International filing date (day/month/year) 11 JANUARY 2000	Priority date (day/month/year) 25 JUNE 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant ISIS PHARMACEUTICALS, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 4 sheets.
- ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).
- These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 16 JANUARY 2001	Date of completion of this report 06 APRIL 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  ANDREW WANG
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/00633

I. Basis of the report

1. With regard to the elements of the international application:*

☒ the international application as originally filed☒ the description:

pages 1-82, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

☒ the claims:

pages 83-84, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of

☒ the drawings:

pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

☒ the sequence listing part of the description:

pages 1-13, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in printed form.
☐ filed together with the international application in computer readable form.
☒ furnished subsequently to this Authority in written form.
☒ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE
☒ the claims, Nos. NONE
☒ the drawings, sheets/fig. NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

**Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.



r

u

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/00633

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. statement**

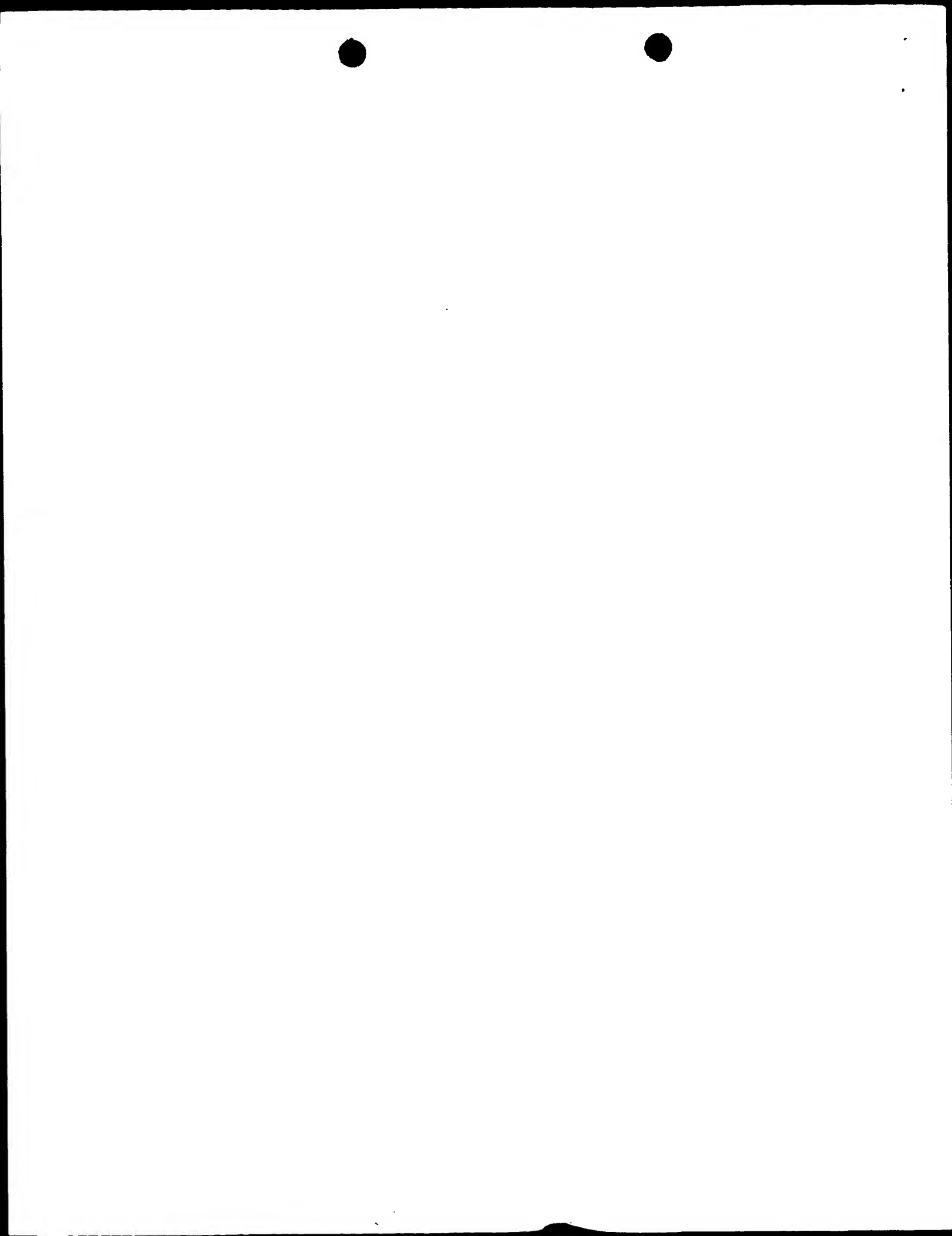
Novelty (N)	Claims <u>1-20</u>	YES
	Claims <u>NONE</u>	NO
Inventive Step (IS)	Claims <u>1-20</u>	YES
	Claims <u>NONE</u>	NO
Industrial Applicability (IA)	Claims <u>1-20</u>	YES
	Claims <u>NONE</u>	NO

2. citations and explanations (Rule 70.7)

Claims 1-20 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest using antisense oligonucleotides comprising 8-30 nucleotides targeted to integrin beta 3. Although two references, authored by Dallabrida et al., were cited as anticipatory references said references, upon further consideration, were not anticipatory since the references teach a vector using the antisense orientation of the encoding transcript and would thus not be embraced by the limitation of 8-30 nucleotides. Therefore, as discussed above, the prior art does not teach or fairly suggest using antisense oligonucleotides 8-30 nucleotides in length targeted to the integrin beta 3 transcript.

----- NEW CITATIONS -----

NONE



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/00633

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

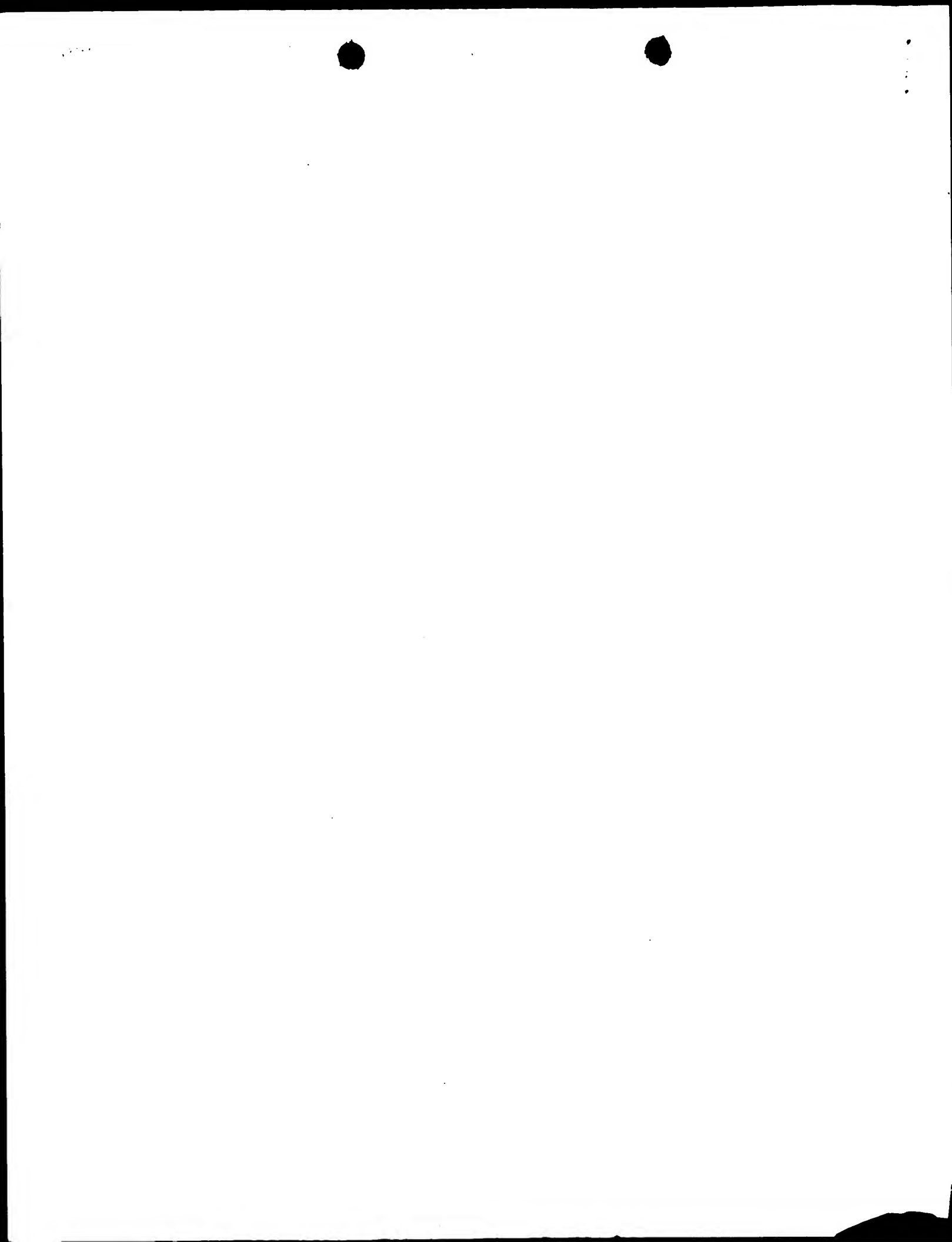
Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): C07H 21/02, 21/04; A61K 48/00; C12N 15/85; C12Q 1/68 and US Cl.: 536/23.1, 24.5; 435/6, 325, 366; 514/44



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/00645 A1

(51) International Patent Classification: C07H 21/02,
21/04, A61K 48/00, C12N 15/85, C12Q 1/68

(74) Agents: LICATA, Jane, Massey et al.; Law Offices of
Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053
(US).

(21) International Application Number: PCT/US00/00633

(22) International Filing Date: 11 January 2000 (11.01.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/344,520 25 June 1999 (25.06.1999) US

(71) Applicant (for all designated States except US): ISIS
PHARMACEUTICALS, INC. [US/US]; 2292 Faraday
Avenue, Carlsbad, CA 92008 (US).

(81) Designated States (national): AE, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE,
ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP,
KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD,
MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD,
SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ,
VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (for US only): BENNETT, C.,
Frank [US/US]; 1347 Cassins Street, Carlsbad, CA 92008
(US). MONIA, Brett, P. [US/US]; 7605 Nueva Castilla
Way, La Costa, CA 92009 (US). COWSERT, Lex, M.
[US/US]; 3008 Newshire Street, Carlsbad, CA 92008
(US).

Published:

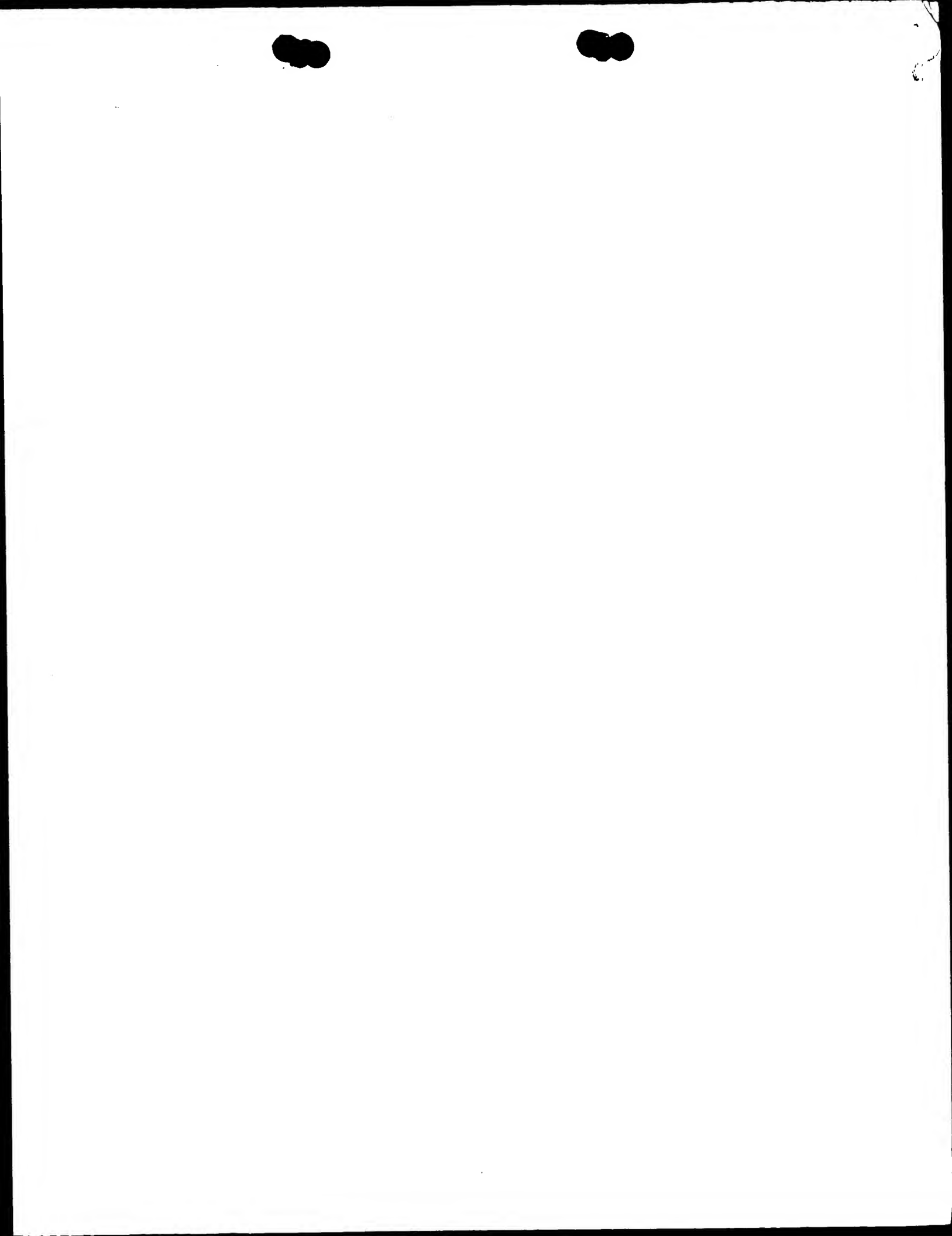
— With international search report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ANTISENSE MODULATION OF INTEGRIN BETA 3 EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of integrin beta 3. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding integrin beta 3. Methods of using these compounds for modulation of integrin beta 3 expression and for treatment of diseases associated with expression of integrin beta 3 are provided.

WO 01/00645 A1



ANTISENSE MODULATION OF INTEGRIN BETA 3 EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and
5 methods for modulating the expression of integrin beta 3.
In particular, this invention relates to antisense
compounds, particularly oligonucleotides, specifically
hybridizable with nucleic acids encoding human integrin
beta 3. Such oligonucleotides have been shown to modulate
10 the expression of integrin beta 3.

BACKGROUND OF THE INVENTION

Cell adhesive contacts are critical for the
development and maintenance of multicellular organisms.
These contacts are mediated by cell adhesion molecules
15 (CAMs), a versatile class of compounds expressed on the
cell surface. Cells adhere to one another and to
extracellular substrates through the concerted action of a
variety of CAMs, which act as both receptors and ligands on
opposing cells. There are four subclasses of CAMs;
20 selectins, cadherins, immunoglobulins and integrins.
Selectins influence the localization of circulating
leukocytes during inflammation, while cadherins and
immunoglobulins establish and maintain cell-to-cell
association and recognition mechanisms (Elangbam et al.,
25 *Vet. Pathol.*, 1997, 34, 61-73). The fourth class of CAMs,
known as integrins, play an important role in cell
migration, cell anchorage to substrates and cytoadhesion
signaling pathways (Akiyama, *Hum. Cell*, 1996, 9, 181-186).

Integrins are heterodimeric cation-dependent membrane
30 glycoproteins composed of an alpha and beta subunit. To
date, 8 beta and 15 alpha subunits have been identified and
these subunits have been shown to combine to form over 20
different $\alpha\beta$ heterodimers. Integrins have been found in
all tissues examined and consist of a large extracellular

domain, a transmembrane domain and a smaller cytoplasmic domain. It is the extracellular domain of the integrin that acts as a receptor for various matrix proteins, while the cytoplasmic domain has been shown to interact with actin filaments of the cytoskeleton, thereby mediating signaling cascades (LaFlamme et al., *Matrix Biol.*, 1997, 16, 153-163).

Integrin beta 3 (also known as human endothelial glycoprotein, GP3A, GPIIIa, ITGB3, CD61 and platelet glycoprotein 3a) is the common beta subunit partner of the members of the $\beta 3$ subfamily of integrins. This subfamily consists of two members, the vitronectin receptor, and the fibrinogen receptor and cells expressing this class of integrin receptor can adhere to various matrix proteins and participate in cytoadhesion driven cellular responses.

Integrin beta 3, in conjunction with integrin alpha v, forms the vitronectin receptor ($\alpha V\beta 3$). This heterodimeric receptor is localized to platelets, endothelial cells, monocytes, macrophages and osteoclasts with the highest expression found in the osteoclasts (Rodan and Rodan, *J. Endocrinol.*, 1997, 154 Suppl, S47-56). The vitronectin receptor functions to mediate the adhesion of cells to vitronectin, and a variety of extracellular matrix proteins. It is through a specific tripeptide sequence referred to as the RGD sequence, so named because of its amino acid composition (arginine-glycine-aspartic acid), that receptor-protein binding occurs. One category of inhibitors that targets the vitronectin receptor are peptidomimetics, designed to block the interactions between the receptor and RGD-containing proteins (Akiyama, *Hum. Cell*, 1996, 9, 181-186; Horton, *Int. J. Biochem. Cell Biol.*, 1997, 29, 721-725).

The activation of the vitronectin receptor has been shown to promote cellular migration and to provide signals

in the regulation of cell proliferation and differentiation and to potentiate the effects of insulin (Ruoslahti, *Kidney Int.*, 1997, 51, 1413-1417). Upregulation of the vitronectin receptor is associated with pathological conditions such as vascular restinosis (Clemetson and Clemetson, *Cell. Mol. Life Sci.*, 1998, 54, 502-513), excessive bone resorption (Rodan and Rodan, *J. Endocrinol.*, 1997, 154 Suppl, S47-56), and the process of angiogenesis during malignant melanomas (Cheresh, *Cancer Metastasis Rev.*, 1991, 10, 3-10).

Integrin beta 3, in conjunction with integrin alpha IIb, also forms the fibrinogen receptor (α IIb/ β 3) which mediates platelet aggregation. This receptor is basally inactive but can be activated by several agonists causing it to bind fibrinogen which then forms cross-bridges to fibrinogen receptors on adjacent cells. This receptor has also been shown to bind other proteins including fibronectin, von Willebrand factor (vWf) and vitronectin (Shattil, *Thromb. Haemost.*, 1993, 70, 224-228).

Mutations in the gene of either subunit of the fibrinogen receptor, resulting in receptor deletion or misfunction, are the primary cause of the bleeding disorder known as Glanzman's thrombasthenia (GT) (Kato, *Crit. Rev. Oncol. Hematol.*, 1997, 26, 1-23). This disorder is transmitted as an autosomal recessive trait and several mutations, including gene deletions, point mutations, and rearrangements have been identified (Kato, *Crit. Rev. Oncol. Hematol.*, 1997, 26, 1-23).

GPIIIa polymorphism is associated with alloantigen phenotype polymorphism known as "Pen polymorphism". Oligonucleotides that hybridize to a polymorphic site at nucleotide 526 on a cDNA encoding GPIIIa, for distinguishing among alleles of GPIIIa, are disclosed in U.S. Patent 5,780,229 and WO 93/01315 (Newman). Also

-4-

disclosed are oligonucleotide primer sets suitable for amplifying a portion of the GPIIIa gene including nucleotide 526 of the GPIIIa cDNA.

Currently, therapeutic agents which affect the function of receptors containing the integrin beta 3 subunit have been designed to interfere with the binding properties of the receptor. As such, several inhibitors have been reported in the art and these include synthetic compounds and their derivatives, antibodies, and peptidomimetics, all of which act as antagonists to receptor ligand binding.

Abciximab, ReoPro[®] (Eli Lilly and Co.) is the Fab fragment of the chimeric human-murine monoclonal antibody 7E3. Abciximab binds to the glycoprotein (GP) IIb/IIIa (α IIb β 3) receptor of human platelets and inhibits platelet aggregation. The mechanism of action is thought to involve steric hindrance and/or conformational effects to block access of large molecules to the receptor rather than direct interaction with the RGD (arginine-glycine-aspartic acid) binding site of GPIIb/IIIa. Abciximab also binds with similar affinity to the vitronectin (α v β 3) receptor found on platelets and vessel wall endothelial and smooth muscle cells.

Abciximab is indicated as an adjunct to percutaneous coronary intervention for the prevention of cardiac ischemic complications. However, a potentially dangerous human antibody response to the chimeric antibody occurred in approximately 6% of patients (Clemetson and Clemetson, *Cell. Mol. Life Sci.*, 1998, 54, 502-513).

Monoclonal antibodies to both integrin beta 3-containing receptors have been reported in the literature. Recently it was demonstrated that prostate carcinoma cells express the α IIb/ β 3 fibrinogen receptor and that antibodies to this receptor were capable of inhibiting the invasive

-5-

properties of the carcinomal cells (Trikha et al., Cancer Res., 1996, 56, 5071-5078).

Monoclonal antibodies produced by three different hybridoma cell lines that target the $\alpha V\beta 3$ (vitronectin) receptor and inhibit the binding of fibronectin or vitronectin to osteoclasts are disclosed in US Patents 5,578,704, 5,652,109 and 5,652,110. These antibodies are used to detect the $\alpha V\beta 3$ integrin and to treat disease conditions characterized by excessive bone resorption (Kim et al., 1997,; Kim et al., 1997,; Kim et al., 1996).

To date, no therapeutic agents that effectively inhibit the expression of integrin beta 3 have been identified. Consequently, there remains a long felt need for additional agents capable of effectively inhibiting integrin beta 3 function by reducing the levels of expression.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of integrin beta 3 expression.

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding integrin beta 3, and which modulate the expression of integrin beta 3. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of integrin beta 3 in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of

-6-

integrin beta 3 by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding integrin beta 3, ultimately modulating the amount of integrin beta 3 produced. This is accomplished by
10 providing antisense compounds which specifically hybridize with one or more nucleic acids encoding integrin beta 3. As used herein, the terms "target nucleic acid" and "nucleic acid encoding integrin beta 3" encompass DNA encoding integrin beta 3, RNA (including pre-mRNA and mRNA)
15 transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically
20 hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein
25 translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of integrin
30 beta 3. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is
35 a preferred target.

-7-

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding integrin beta 3. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set

-8-

of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene
5 encoding integrin beta 3, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the
10 corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e.,
15 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a
20 translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted
25 effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation
30 initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination
35 codon and 3' end of an mRNA or corresponding nucleotides on

the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself
5 as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is
10 translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where
15 aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and
20 therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently
25 well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between
30 complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a
35 nucleotide at a certain position of an oligonucleotide is

-10-

capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the

5 DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a

10 sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its

15 target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient

20 degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in

25 vitro assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression

30 with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore,

35 been harnessed for research use.

-11-

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 25 nucleobases. As is known in the art, a nucleoside is a base-sugar combination.

-12-

The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a

5 phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently

10 link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure,

15 the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds

20 useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone

25 and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

30 Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates,

35 phosphinates, phosphoramidates including 3'-amino

-13-

phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are

-14-

not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444;
5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564;
5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677;
5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;
5 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;
5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of
which are commonly owned with this application, and each of
which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the
10 sugar and the internucleoside linkage, i.e., the backbone,
of the nucleotide units are replaced with novel groups.
The base units are maintained for hybridization with an
appropriate nucleic acid target compound. One such
oligomeric compound, an oligonucleotide mimetic that has
15 been shown to have excellent hybridization properties, is
referred to as a peptide nucleic acid (PNA). In PNA
compounds, the sugar-backbone of an oligonucleotide is
replaced with an amide containing backbone, in particular
an aminoethylglycine backbone. The nucleobases are
20 retained and are bound directly or indirectly to aza
nitrogen atoms of the amide portion of the backbone.
Representative United States patents that teach the
preparation of PNA compounds include, but are not limited
to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of
25 which is herein incorporated by reference. Further teaching
of PNA compounds can be found in Nielsen et al., *Science*,
1991, 254, 1497-1500.

Most preferred embodiments of the invention are
oligonucleotides with phosphorothioate backbones and
30 oligonucleosides with heteroatom backbones, and in
particular $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$ [known as a
methylene (methylimino) or MMI backbone], $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$,
 $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$ and $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$ [wherein the
native phosphodiester backbone is represented as $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$
35] of the above referenced U.S. patent 5,489,677, and the

-15-

amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

5 Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and
10 alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the
15 following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA
20 cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred
25 modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-
30 dimethylaminoethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-

35

-16-

F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.:

10 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878;
5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;
5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053;
5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920,
15 certain of which are commonly owned with the instant
application, and each of which is herein incorporated by
reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural"

20 nucleobases include the purine bases adenine (A) and
guanine (G), and the pyrimidine bases thymine (T), cytosine
(C) and uracil (U). Modified nucleobases include other
synthetic and natural nucleobases such as 5-methylcytosine
(5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine,
25 2-aminoadenine, 6-methyl and other alkyl derivatives of
adenine and guanine, 2-propyl and other alkyl derivatives
of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-
thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil
and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil
30 (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-
thioalkyl, 8-hydroxyl and other 8-substituted adenines and
guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl
and other 5-substituted uracils and cytosines, 7-
methylguanine and 7-methyladenine, 8-azaguanine and 8-
35 azaadenine, 7-deazaguanine and 7-deazaadenine and 3-

-17-

deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993.

Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

-18-

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;

-19-

5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;
4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582;
4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;
5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;
5 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,
5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667;
5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481;
5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and
5,688,941, certain of which are commonly owned with the
10 instant application, and each of which is herein
incorporated by reference.

It is not necessary for all positions in a given
compound to be uniformly modified, and in fact more than
one of the aforementioned modifications may be incorporated
15 in a single compound or even at a single nucleoside within
an oligonucleotide. The present invention also includes
antisense compounds which are chimeric compounds.
"Chimeric" antisense compounds or "chimeras," in the
context of this invention, are antisense compounds,
20 particularly oligonucleotides, which contain two or more
chemically distinct regions, each made up of at least one
monomer unit, i.e., a nucleotide in the case of an
oligonucleotide compound. These oligonucleotides typically
contain at least one region wherein the oligonucleotide is
25 modified so as to confer upon the oligonucleotide increased
resistance to nuclease degradation, increased cellular
uptake, and/or increased binding affinity for the target
nucleic acid. An additional region of the oligonucleotide
may serve as a substrate for enzymes capable of cleaving
30 RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is
a cellular endonuclease which cleaves the RNA strand of an
RNA:DNA duplex. Activation of RNase H, therefore, results
in cleavage of the RNA target, thereby greatly enhancing
the efficiency of oligonucleotide inhibition of gene
35 expression. Consequently, comparable results can often be

-20-

obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely
5 detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more
10 oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid
15 structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is
20 herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors
25 including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

30 The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules. The compounds of the invention may
35 also be admixed, encapsulated, conjugated or otherwise

-21-

associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

-22-

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric

acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids

-24-

such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

10 The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the
15 expression of integrin beta 3 is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically
20 acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

25 The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding integrin beta 3, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid
30 encoding integrin beta 3 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of
35 integrin beta 3 in a sample may also be prepared.

-25-

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not

-26-

limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and
5 liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present
10 invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s)
15 or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

20 The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in
25 aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

30 In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature
35 these formulations vary in the components and the

-27-

consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily

-28-

phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion.

Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y.,

-29-

volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York,

-30-

N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include
5 naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for
10 example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external
15 phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly
20 used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the
25 formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid,
30 and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
35 and Banker (Eds.), 1988, Marcel Dekker, Inc., New York,

-31-

N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is

-32-

of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules

5 (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how

10 to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York,

15 N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

20 Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol

25 monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain

30 alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared

35 without the use of cosurfactants and alcohol-free self-

emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives
5 of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides,
10 saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w
15 and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of
20 improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms,
25 improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be
30 particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion

-34-

compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess

-35-

the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages in vivo.

5 In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to
10 pass through such fine pores.

 Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect
15 encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome
20 formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

 Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological
25 membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

30 Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-
35 effects related to high systemic absorption of the

-36-

administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

5 Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications
10 resulted in the targeting of the upper epidermis.

 Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds
15 to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

20 Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous
25 interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, 1992, 19, 269-274).

30 One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic
35 liposome compositions generally are formed from dimyristoyl

-37-

phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced

-38-

circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1} , or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765). Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside G_{M1} , galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C₁₂15G, that contains a

PEG moiety. Illum et al. (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klivanov et al. (*FEBS Lett.*, 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight

-40-

nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

-41-

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

-42-

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

5

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate,

polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm.*

5 *Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid,
10 linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C₁₋₁₀ alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate,
15 laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990,
20 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and
25 fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term
30 "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium

-44-

dehydrocholate), deoxycholic acid (sodium deoxycholate),
glucholic acid (sodium glucholate), glycholic acid (sodium
glycocholate), glycodeoxycholic acid (sodium
glycodeoxycholate), taurocholic acid (sodium taurocholate),
5 taurodeoxycholic acid (sodium taurodeoxycholate),
chenodeoxycholic acid (sodium chenodeoxycholate),
ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-
fusidate (STDHF), sodium glycodihydrofusidate and
polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical*
10 *Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92;
Swinyard, Chapter 39 In: *Remington's Pharmaceutical*
Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co.,
Easton, PA, 1990, pages 782-783; Muranishi, *Critical*
Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33;
15 Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25;
Yamashita et al., *J. Pharm. Sci.*, 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in
connection with the present invention, can be defined as
20 compounds that remove metallic ions from solution by
forming complexes therewith, with the result that
absorption of oligonucleotides through the mucosa is
enhanced. With regards to their use as penetration
enhancers in the present invention, chelating agents have
25 the added advantage of also serving as DNase inhibitors, as
most characterized DNA nucleases require a divalent metal
ion for catalysis and are thus inhibited by chelating
agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315-339).
Chelating agents of the invention include but are not
30 limited to disodium ethylenediaminetetraacetate (EDTA),
citric acid, salicylates (e.g., sodium salicylate, 5-
methoxysalicylate and homovanilate), N-acyl derivatives of
collagen, laureth-9 and N-amino acyl derivatives of beta-
diketones (enamines) (Lee et al., *Critical Reviews in*

Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51).

5 Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through
10 the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in*
15 *Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the
20 cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al.,
25 PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol,
30 pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also

-46-

incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity *per se*) but is recognized
5 as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier
10 compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a
15 common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al.,
20 *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical
25 carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in
30 mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch,

polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

-48-

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine,

-49-

cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 1206-1228). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and

-50-

can generally be estimated based on EC_{50} s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, 5 monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be 10 desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

15 While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

20 **EXAMPLES**

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites

2' -Deoxy and 2' -methoxy beta-cyanoethyldiisopropyl 25 phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides 30 synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine 35 (5-Me-C) nucleotides were synthesized according to

-51-

published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

5 **2'-Fluoro amidites**

2'-Fluorodeoxyadenosine amidites

 2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

 The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups.

-52-

Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

10 2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

20 2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum

-53-

was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70%

-54-

product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in CHCl_3 (1.5 L) and extracted with 2x500 mL of saturated NaHCO_3 and 2x500 mL of saturated NaCl . The organic phase was dried over Na_2SO_4 ,
5 filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc /hexane/acetone (5:5:1) containing 0.5% Et_3NH . The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional
10 was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine
15 (106 g, 0.167 M), DMF /pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the
20 addition of MeOH . Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C . The residue was dissolved in CHCl_3 (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl . The water
25 layers were back extracted with 200 mL of CHCl_3 . The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc /hexane(4:1). Pure product fractions were
30 evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-
35 acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-

-55-

methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl_3 was added dropwise, over a 30 minute period, to the stirred solution maintained at $0-10^\circ\text{C}$, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO_3 and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-

-56-

cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl_3 (700 mL) and extracted with saturated NaHCO_3 (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO_4 and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO_3 (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over MgSO_4 and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside

-57-

amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O²-2'-anhydro-5-methyluridine

O²-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until

-58-

the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-0²-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and
5 heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (R_f 0.67 for desired product and R_f 0.82 for ara-T side product, ethyl acetate) indicated about 70%
10 conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once
15 the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate
20 fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99%
25 pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with
30 triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P₂O₅ under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a
35 clear solution. Diethyl-azodicarboxylate (6.98mL,

-59-

44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred
5 for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-
10 butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-
15 5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH₂Cl₂ (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH₂Cl₂ and the combined organic phase was washed with water, brine and
20 dried over anhydrous Na₂SO₄. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under
25 vacuum; residue chromatographed to get 5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

30 5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this

-60-

solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH₂Cl₂). Aqueous NaHCO₃ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na₂SO₄, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO₃ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH₂Cl₂ to get 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminoxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH₂Cl₂). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH₂Cl₂ to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

-61-

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N¹,N¹-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

-62-

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs.

5 Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

10 The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor
15 amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures
20 should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-
25 5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-
30 dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also

known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH₂-O-CH₂-N(CH₂)₂, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

5 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O²-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

25 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH₂Cl₂ (2x200 mL). The combined CH₂Cl₂ layers are washed with saturated NaHCO₃ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel

-64-

chromatography using MeOH:CH₂Cl₂:Et₃N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH₂Cl₂ (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

-65-

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023,

-66-

5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

10 PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in *Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric

Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligo-

-67-

nucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphor-
5 amidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The
10 fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect
15 all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25
20 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate

25 Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.
30

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phos-

-68-

phorothioate]--[2'-O-(methoxyethyl) phosphodiester]
chimeric oligonucleotides are prepared as per the above
procedure for the 2'-O-methyl chimeric oligonucleotide with
the substitution of 2'-O-(methoxyethyl) amidites for the
5 2'-O-methyl amidites, oxidization with iodine to generate
the phosphodiester internucleotide linkages within the
wing portions of the chimeric structures and sulfurization
utilizing 3,4-dithiolane-2-one 1,1 dioxide (Beaucage
Reagent) to generate the phosphorothioate internucleotide
10 linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleo-
sides and mixed chimeric oligonucleotides/oligonucleosides
are synthesized according to United States patent
5,623,065, herein incorporated by reference.

15 Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column
(Applied Biosystems) and deblocking in concentrated
ammonium hydroxide at 55°C for 18 hours, the
20 oligonucleotides or oligonucleosides are purified by
precipitation twice out of 0.5 M NaCl with 2.5 volumes
ethanol. Synthesized oligonucleotides were analyzed by
polyacrylamide gel electrophoresis on denaturing gels and
judged to be at least 85% full length material. The
25 relative amounts of phosphorothioate and phosphodiester
linkages obtained in synthesis were periodically checked by
³¹P nuclear magnetic resonance spectroscopy, and for some
studies oligonucleotides were purified by HPLC, as
described by Chiang et al., *J. Biol. Chem.* 1991, 266,
30 18162-18171. Results obtained with HPLC-purified material
were similar to those obtained with non-HPLC purified
material.

Example 7**Oligonucleotide Synthesis - 96 Well Plate Format**

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dithiolane-2-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyl-diisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8**Oligonucleotide Analysis - 96 Well Plate Format**

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test

-70-

plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

5 **Example 9**

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at
10 measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

15 **T-24 cells:**

The transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life
20 Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and
25 dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates
30 and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained
35 from the American Type Culture Collection (ATCC) (Manassas,

-71-

VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

10 NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of OPTI-MEM™-1 containing 3.75 μ g/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was

-72-

replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

Example 10

Analysis of oligonucleotide inhibition of integrin beta 3 expression

Antisense modulation of integrin beta 3 expression can be assayed in a variety of ways known in the art. For example, integrin beta 3 mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

integrin beta 3 protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to integrin beta 3 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of

polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997.

Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc.,

-74-

Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 100 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack

-75-

containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 μ L water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 μ L water.

5 The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are
10 carried out.

Example 13

Real-time Quantitative PCR Analysis of integrin beta 3 mRNA Levels

Quantitation of integrin beta 3 mRNA levels was
15 determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput
20 quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by
25 including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City,
30 CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by
35 the proximity of the 3' quencher dye. During

-76-

amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl₂, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). integrin beta 3 probes and primers were designed to hybridize to the human integrin beta 3 sequence, using published sequence information (GenBank accession number J02703, incorporated herein as SEQ ID NO:1).

For integrin beta 3 the PCR primers were:
forward primer: TTTACCACTGATGCCAAGACTCA (SEQ ID NO: 2)

-77-

reverse primer: CCGTCATTAGGCTGGACAATG (SEQ ID NO: 3) and
the PCR probe was: FAM-ATAGCATTGGACGGAAGGCTGGCAG-TAMRA
(SEQ ID NO: 4) where FAM (PE-Applied Biosystems, Foster
City, CA) is the fluorescent reporter dye) and TAMRA (PE-
5 Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 5)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 6) and the
PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ
10 ID NO: 7) where JOE (PE-Applied Biosystems, Foster City,
CA) is the fluorescent reporter dye) and TAMRA (PE-Applied
Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of integrin beta 3 mRNA levels

15 Eighteen hours after antisense treatment, cell
monolayers were washed twice with cold PBS and lysed in 1
mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA
was prepared following manufacturer's recommended
protocols. Twenty micrograms of total RNA was fractionated
20 by electrophoresis through 1.2% agarose gels containing
1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc.
Solon, OH). RNA was transferred from the gel to HYBOND™-N+
nylon membranes (Amersham Pharmacia Biotech, Piscataway,
NJ) by overnight capillary transfer using a
25 Northern/Southern Transfer buffer system (TEL-TEST "B"
Inc., Friendswood, TX). RNA transfer was confirmed by UV
visualization. Membranes were fixed by UV cross-linking
using a STRATALINKER™ UV Crosslinker 2400 (Stratagene,
Inc, La Jolla, CA).

30 Membranes were probed using QUICKHYB™ hybridization
solution (Stratagene, La Jolla, CA) using manufacturer's
recommendations for stringent conditions with a integrin
beta 3 specific probe prepared by PCR using the forward
primer TTTACCACTGATGCCAAGACTCA (SEQ ID NO: 2) and the
35 reverse primer CCGTCATTAGGCTGGACAATG (SEQ ID NO: 3). To

-78-

normalize for variations in loading and transfer efficiency membranes were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA). Hybridized membranes were visualized and quantitated
 5 using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

Antisense inhibition of integrin beta 3 expression-
 10 **phosphorothioate oligodeoxynucleotides**

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human integrin beta 3 RNA, using published sequences (GenBank accession number J02703, incorporated herein as
 15 SEQ ID NO: 1). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. J02703), to which the oligonucleotide binds. All compounds in Table 1 are oligodeoxynucleotides with phosphorothioate
 20 backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on integrin beta 3 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

25

Table 1

Inhibition of integrin beta 3 mRNA levels by
 phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET	SEQUENCE	% Inhibition	SEQ ID NO.
		SITE			
25177	Start Codon	7	gcatctcgtccgcctccc	56	8
25178	Coding	50	gccagcacagtcacccag	0	9
25179	Coding	258	actcactgggaactcgat	34	10
25180	Coding	389	tggatggagaaattcttc	36	11
35 25181	Coding	480	gttctggatgctccacag	45	12

-79-

	25182	Coding	545	gccccgaagccaatccgc	0	13
	25183	Coding	689	aagcgggtcacctggtca	0	14
	25184	Coding	802	cattcctccagccaatct	40	15
	25185	Coding	863	cttccgtccaatgctata	4	16
5	25186	Coding	916	tgtcactaccaacatgac	68	17
	25187	Coding	977	agcttctcagtcacagc	72	18
	25188	Coding	1033	agagattgactacatttt	7	19
	25189	Coding	1144	ctttagaacggattttcc	0	20
	25190	Coding	1182	agacaactcttcagggag	38	21
10	25191	Coding	1267	tcaccgtgtctccaatct	47	22
	25192	Coding	1339	ccacgggctttatggtaa	37	23
	25193	Coding	1406	tcagcttgggcctggcag	40	24
	25194	Coding	1513	agtcctcctctgagcact	45	25
	25195	Coding	1600	cacattgaccacagaggc	26	26
15	25196	Coding	1660	agaagtcgtcacactcgc	28	27
	25197	Coding	1798	gcccatgtgtggacatgc	0	28
	25198	Coding	1903	aggcatctgggcaggtgg	10	29
	25199	Coding	1943	tcaaacttcttacactcc	46	30
	25200	Coding	2036	gcataccttgccagtgtcc	53	31
20	25201	Coding	2075	acgacacagtcatacctca	0	32
	25202	Coding	2126	accacatacaggatggac	0	33
	25203	Coding	2240	gtgatgaggagtctccag	2	34
	25204	Coding	2302	tgtcccattttgctctgg	47	35
	25205	Coding	2342	aaggtagacgtggcctct	29	36
25	25206	Stop Codon	2384	atgactgcttatcattaa	0	37
	25207	3' UTR	2424	gactcctgcaatcctggc	47	38
	25208	3' UTR	2510	acatactgacattctccc	0	39
	25209	3' UTR	2564	acacacacataaacacac	0	40
	25210	3' UTR	2679	tcaccctcaagctaagca	0	41
30	25211	3' UTR	2719	tctcactgaggtaatgaa	34	42
	25212	3' UTR	2859	agctgctgagagtcaggc	78	43
	25213	3' UTR	2934	tccttcggcctgagggag	43	44
	25214	3' UTR	2976	aaaggcacaggcagctct	0	45
	25215	3' UTR	3108	agatgctggtacatcccc	60	46
35	25216	3' UTR	3147	caataaggcaaatctgaa	23	47

As shown in Table 1, SEQ ID NOs 8, 10, 11, 12, 15, 17, 18, 21, 22, 23, 24, 25, 26, 27, 30, 31, 35, 36, 38, 42, 43, 44, 46 and 47 demonstrated at least 20% inhibition of integrin beta 3 expression in this assay and are therefore preferred.

-80-

Example 16:**Antisense inhibition of integrin beta 3 expression-
phosphorothioate 2'-MOE gapmer oligonucleotides**

In accordance with the present invention, a second
5 series of oligonucleotides targeted to human integrin beta
3 were synthesized. The oligonucleotide sequences are
shown in Table 2. Target sites are indicated by nucleotide
numbers, as given in the sequence source reference (Genbank
accession no. J02703), to which the oligonucleotide binds.

10 All compounds in Table 2 are chimeric oligonucleotides
("gapmers") 18 nucleotides in length, composed of a central
"gap" region consisting of ten 2'-deoxynucleotides, which
is flanked on both sides (5' and 3' directions) by four-
nucleotide "wings". The wings are composed of 2'-
15 methoxyethyl (2'-MOE)nucleotides. The internucleoside
(backbone) linkages are phosphorothioate (P=S) throughout
the oligonucleotide. Cytidine residues in the 2'-MOE wings
are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as
20 described in other examples herein and are averaged from
two experiments. If present, "N.D." indicates "no data".

Table 2

Inhibition of integrin beta 3 mRNA levels by chimeric
25 phosphorothioate oligonucleotides having 2'-MOE wings and a
deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
25217	Start Codon	7	gcatctcggtccgcctccc	17	8
30 25218	Coding	50	gccagcacagtcacccag	49	9
25219	Coding	258	actcactgggaactcgat	0	10
25220	Coding	389	tggatggagaaattcttc	33	11
25221	Coding	480	gttctggatgctccacag	58	12
25222	Coding	545	gccccgaagccaatccgc	35	13
35 25223	Coding	689	aagcgggtcacctggtca	0	14
25224	Coding	802	cattcctccagccaatct	0	15

-81-

	25225	Coding	863	cttccgtccaatgctata	0	16
	25226	Coding	916	tgtcactaccaacatgac	3	17
	25227	Coding	977	agcttctcagtcacagc	0	18
	25228	Coding	1033	agagattgactacatttt	0	19
5	25229	Coding	1144	ctttagaacggattttcc	30	20
	25230	Coding	1182	agacaactcttcagggag	5	21
	25231	Coding	1267	tcaccgtgtctccaatct	0	22
	25232	Coding	1339	ccacgggctttatggtaa	28	23
	25233	Coding	1406	tcagcttgggcctggcag	0	24
10	25234	Coding	1513	agtcctcctctgagcact	35	25
	25235	Coding	1600	cacattgaccacagaggc	0	26
	25236	Coding	1660	agaagtcgtcacactcgc	63	27
	25237	Coding	1798	gcccattgctggacatgc	80	28
	25238	Coding	1903	aggcatctgggcaggtgg	48	29
15	25239	Coding	1943	tcaaacttcttacactcc	22	30
	25240	Coding	2036	gcatccttgccagtgtcc	0	31
	25241	Coding	2075	acgacacagtcacacctca	0	32
	25242	Coding	2126	accacatacaggatggac	58	33
	25243	Coding	2240	gtgatgaggagtttccag	8	34
20	25244	Coding	2302	tgtcccattttgctctgg	56	35
	25245	Coding	2342	aaggtagacgtggcctct	45	36
	25246	Stop	2384	atgactgcttatcattaa	11	37
		Codon				
	25247	3' UTR	2424	gactcctgcaatcctggc	8	38
	25248	3' UTR	2510	acatactgacattctccc	0	39
25	25249	3' UTR	2564	acacacacataaacacac	0	40
	25250	3' UTR	2679	tcaccctcaagctaagca	52	41
	25251	3' UTR	2719	tctcactgaggtaatgaa	19	42
	25252	3' UTR	2859	agctgctgagagtcaggc	0	43
	25253	3' UTR	2934	tccttcggcctgagggag	23	44
30	25254	3' UTR	2976	aaaggcacaggcagctct	0	45
	25255	3' UTR	3108	agatgctgggtacatcccc	0	46
	25256	3' UTR	3147	caataaggcaaatctgaa	0	47

As shown in Table 2, SEQ ID NOs 9, 11, 12, 13, 20, 23, 25, 27, 28, 29, 30, 33, 35, 36, 41 and 44 demonstrated at least 20% inhibition of integrin beta 3 expression in this experiment and are therefore preferred.

Example 17

Western blot analysis of integrin beta 3 protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS,

-82-

suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to

5 integrin beta 3 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

-83-

What is claimed is:

1. An antisense compound 8 to 30 nucleobases in length targeted to a nucleic acid molecule encoding human integrin beta 3, wherein said antisense compound
5 specifically hybridizes with and inhibits the expression of human integrin beta 3.
2. The antisense compound of claim 1 which is an antisense oligonucleotide.
3. The antisense compound of claim 2 wherein the
10 antisense oligonucleotide has a sequence comprising SEQ ID NO: 8, 10, 11, 12, 15, 17, 18, 21, 22, 23, 24, 25, 26, 27, 30, 31, 35, 36, 38, 42, 43, 44, 46, 47, 9, 13, 20, 28, 29, 33 or 41.
4. The antisense compound of claim 2 wherein the
15 antisense oligonucleotide has a sequence comprising SEQ ID NO: 11, 12, 23, 25, 27, 30, 35, 36 or 44.
5. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.
- 20 6. The antisense compound of claim 5 wherein the modified internucleoside linkage is a phosphorothioate linkage.
7. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified
25 sugar moiety.
8. The antisense compound of claim 7 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.
9. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified
30 nucleobase.
10. The antisense compound of claim 9 wherein the modified nucleobase is a 5-methylcytosine.
11. The antisense compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

-84-

12. A composition comprising the antisense compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

5 14. The composition of claim 12 wherein the antisense compound is an antisense oligonucleotide.

15 15. A method of inhibiting the expression of integrin beta 3 in human cells or tissues comprising contacting said cells or tissues with the antisense
10 compound of claim 1 so that expression of integrin beta 3 is inhibited.

15 16. A method of treating a human having a disease or condition associated with integrin beta 3 comprising administering to said animal a therapeutically or
15 prophylactically effective amount of the antisense compound of claim 1 so that expression of integrin beta 3 is inhibited.

17. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

20 18. The method of claim 17 wherein the hyperproliferative disorder is cancer.

19. The method of claim 17 wherein the hyperproliferative disorder is vascular stenosis or restenosis.

25 20. The method of claim 16 wherein the disease or condition is a bone resorption disorder.

SEQUENCE LISTING

<110> C. Frank Bennett
 Brett P. Monia
 Lex M. Cowser
 ISIS PHARMACEUTICALS, INC.

<120> ANTISENSE MODULATION OF INTEGRIN BETA 3 EXPRESSION

<130> RTSP-0047

<150> US 09/344,520

<151> 1999-06-25

<160> 47

<210> 1

<211> 3170

<212> DNA

<213> Homo sapiens

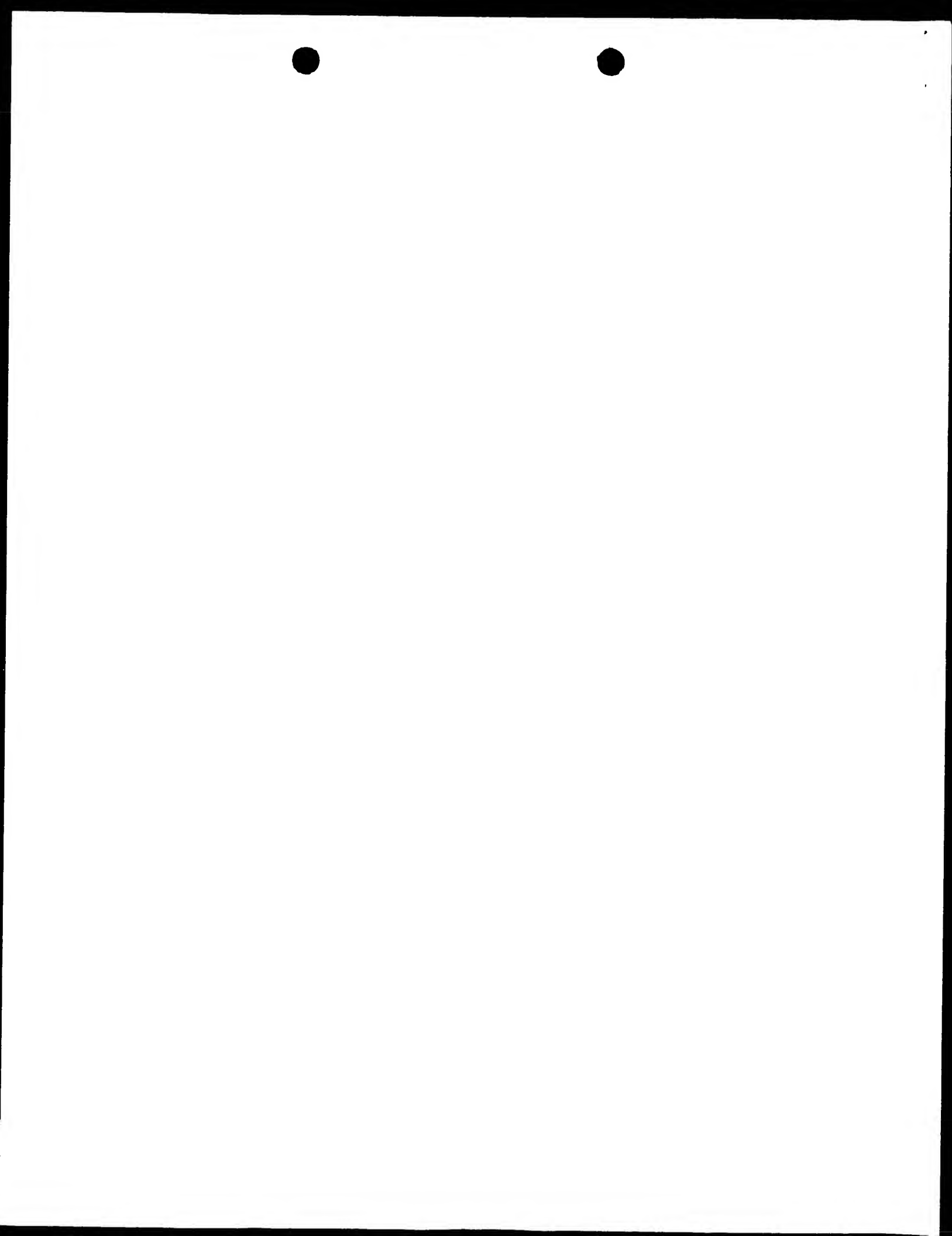
<220>

<221> CDS

<222> (21)..(2387)

<400> 1

cgccgcggga ggcggacgag atg cga gcg cgg ccg cgg ccc cgg ccg ctc	50
Met Arg Ala Arg Pro Arg Pro Arg Pro Leu	
1 5 10	
tgg gtg act gtg ctg gcg ctg ggg gcg ctg gcg ggc gtt ggc gta gga	98
Trp Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly	
15 20 25	
ggg ccc aac atc tgt acc acg cga ggt gtg agc tcc tgc cag cag tgc	146
Gly Pro Asn Ile Cys Thr Thr Arg Gly Val Ser Ser Cys Gln Gln Cys	
30 35 40	
ctg gct gtg agc ccc atg tgt gcc tgg tgc tct gat gag gcc ctg cct	194
Leu Ala Val Ser Pro Met Cys Ala Trp Cys Ser Asp Glu Ala Leu Pro	
45 50 55	
ctg ggc tca cct cgc tgt gac ctg aag gag aat ctg ctg aag gat aac	242
Leu Gly Ser Pro Arg Cys Asp Leu Lys Glu Asn Leu Leu Lys Asp Asn	
60 65 70	
tgt gcc cca gaa tcc atc gag ttc cca gtg agt gag gcc cga gta cta	290
Cys Ala Pro Glu Ser Ile Glu Phe Pro Val Ser Glu Ala Arg Val Leu	
75 80 85 90	
gag gac agg ccc ctc agc gac aag ggc tct gga gac agc tcc cag gtc	338
Glu Asp Arg Pro Leu Ser Asp Lys Gly Ser Gly Asp Ser Ser Gln Val	
95 100 105	
act caa gtc agt ccc cag agg att gca ctc cgg ctc cgg cca gat gat	386
Thr Gln Val Ser Pro Gln Arg Ile Ala Leu Arg Leu Arg Pro Asp Asp	
110 115 120	



tcg aag aat ttc tcc atc caa gtg cgg cag gtg gag gat tac cct gtg	434
Ser Lys Asn Phe Ser Ile Gln Val Arg Gln Val Glu Asp Tyr Pro Val	
125 130 135	
gac atc tac tac ttg atg gac ctg tct tac tcc atg aag gat gat ctg	482
Asp Ile Tyr Tyr Leu Met Asp Leu Ser Tyr Ser Met Lys Asp Asp Leu	
140 145 150	
tgg agc atc cag aac ctg ggt acc aag ctg gcc acc cag atg cga aag	530
Trp Ser Ile Gln Asn Leu Gly Thr Lys Leu Ala Thr Gln Met Arg Lys	
155 160 165 170	
ctc acc agt aac ctg cgg att ggc ttc ggg gca ttt gtg gac aag cct	578
Leu Thr Ser Asn Leu Arg Ile Gly Phe Gly Ala Phe Val Asp Lys Pro	
175 180 185	
gtg tca cca tac atg tat atc tcc cca cca gag gcc ctc gaa aac ccc	626
Val Ser Pro Tyr Met Tyr Ile Ser Pro Pro Glu Ala Leu Glu Asn Pro	
190 195 200	
tgc tat gat atg aag acc acc tgc ttg ccc atg ttt ggc tac aaa cac	674
Cys Tyr Asp Met Lys Thr Thr Cys Leu Pro Met Phe Gly Tyr Lys His	
205 210 215	
gtg ctg acg cta act gac cag gtg acc cgc ttc aat gag gaa gtg aag	722
Val Leu Thr Leu Thr Asp Gln Val Thr Arg Phe Asn Glu Glu Val Lys	
220 225 230	
aag cag agt gtg tca cgg aac cga gat gcc cca gag ggt ggc ttt gat	770
Lys Gln Ser Val Ser Arg Asn Arg Asp Ala Pro Glu Gly Gly Phe Asp	
235 240 245 250	
gcc atc atg cag gct aca gtc tgt gat gaa aag att ggc tgg agg aat	818
Ala Ile Met Gln Ala Thr Val Cys Asp Glu Lys Ile Gly Trp Arg Asn	
255 260 265	
gat gca tcc cac ttg ctg gtg ttt acc act gat gcc aag act cat ata	866
Asp Ala Ser His Leu Leu Val Phe Thr Thr Asp Ala Lys Thr His Ile	
270 275 280	
gca ttg gac gga agg ctg gca ggc att gtc cag cct aat gac ggg cag	914
Ala Leu Asp Gly Arg Leu Ala Gly Ile Val Gln Pro Asn Asp Gly Gln	
285 290 295	
tgt cat gtt ggt agt gac aat cat tac tct gcc tcc act acc atg gat	962
Cys His Val Gly Ser Asp Asn His Tyr Ser Ala Ser Thr Thr Met Asp	
300 305 310	
tat ccc tct ttg ggg ctg atg act gag aag cta tcc cag aaa aac atc	1010
Tyr Pro Ser Leu Gly Leu Met Thr Glu Lys Leu Ser Gln Lys Asn Ile	
315 320 325 330	
aat ttg atc ttt gca gtg act gaa aat gta gtc aat ctc tat cag aac	1058
Asn Leu Ile Phe Ala Val Thr Glu Asn Val Val Asn Leu Tyr Gln Asn	
335 340 345	
tat agt gag ctc atc cca ggg acc aca gtt ggg gtt ctg tcc atg gat	1106
Tyr Ser Glu Leu Ile Pro Gly Thr Thr Val Gly Val Leu Ser Met Asp	
350 355 360	

tcc agc aat gtc ctc cag ctc att gtt gat gct tat ggg aaa atc cgt	1154
Ser Ser Asn Val Leu Gln Leu Ile Val Asp Ala Tyr Gly Lys Ile Arg	
365 370 375	
tct aaa gtc gag ctg gaa gtg cgt gac ctc cct gaa gag ttg tct cta	1202
Ser Lys Val Glu Leu Glu Val Arg Asp Leu Pro Glu Glu Leu Ser Leu	
380 385 390	
tcc ttc aat gcc acc tgc ctc aac aat gag gtc atc cct ggc ctc aag	1250
Ser Phe Asn Ala Thr Cys Leu Asn Asn Glu Val Ile Pro Gly Leu Lys	
395 400 405 410	
tct tgt atg gga ctc aag att gga gac acg gtg agc ttc agc att gag	1298
Ser Cys Met Gly Leu Lys Ile Gly Asp Thr Val Ser Phe Ser Ile Glu	
415 420 425	
gcc aag gtg cga ggc tgt ccc cag gag aag gag aag tcc ttt acc ata	1346
Ala Lys Val Arg Gly Cys Pro Gln Glu Lys Glu Lys Ser Phe Thr Ile	
430 435 440	
aag ccc gtg ggc ttc aag gac agc ctg atc gtc cag gtc acc ttt gat	1394
Lys Pro Val Gly Phe Lys Asp Ser Leu Ile Val Gln Val Thr Phe Asp	
445 450 455	
tgt gac tgt gcc tgc cag gcc caa gct gaa cct aat agc cat cgc tgc	1442
Cys Asp Cys Ala Cys Gln Ala Gln Ala Glu Pro Asn Ser His Arg Cys	
460 465 470	
aac aat ggc aat ggg acc ttt gag tgt ggg gta tgc cgt tgt ggg cct	1490
Asn Asn Gly Asn Gly Thr Phe Glu Cys Gly Val Cys Arg Cys Gly Pro	
475 480 485 490	
ggc tgg ctg gga tcc cag tgt gag tgc tca gag gag gac tat cgc cct	1538
Gly Trp Leu Gly Ser Gln Cys Glu Cys Ser Glu Glu Asp Tyr Arg Pro	
495 500 505	
tcc cag cag gac gag tgc agc ccc cga gag ggt cag ccc gtc tgc agc	1586
Ser Gln Gln Asp Glu Cys Ser Pro Arg Glu Gly Gln Pro Val Cys Ser	
510 515 520	
cag cgg ggc gag tgc ctc tgt ggt caa tgt gtc tgc cac agc agt gac	1634
Gln Arg Gly Glu Cys Leu Cys Gly Gln Cys Val Cys His Ser Ser Asp	
525 530 535	
ttt ggc aag atc acg ggc aag tac tgc gag tgt gac gac ttc tcc tgt	1682
Phe Gly Lys Ile Thr Gly Lys Tyr Cys Glu Cys Asp Asp Phe Ser Cys	
540 545 550	
gtc cgc tac aag ggg gag atg tgc tca ggc cat ggc cag tgc agc tgt	1730
Val Arg Tyr Lys Gly Glu Met Cys Ser Gly His Gly Gln Cys Ser Cys	
555 560 565 570	
ggg gac tgc ctg tgt gac tcc gac tgg acc ggc tac tac tgc aac tgt	1778
Gly Asp Cys Leu Cys Asp Ser Asp Trp Thr Gly Tyr Tyr Cys Asn Cys	
575 580 585	
acc acg cgt act gac acc tgc atg tcc agc aat ggg ctg ctg tgc agc	1826
Thr Thr Arg Thr Asp Thr Cys Met Ser Ser Asn Gly Leu Leu Cys Ser	
590 595 600	

ggc cgc ggc aag tgt gaa tgt ggc agc tgt gtc tgt atc cag ccg ggc 1874
 Gly Arg Gly Lys Cys Glu Cys Gly Ser Cys Val Cys Ile Gln Pro Gly
 605 610 615

tcc tat ggg gac acc tgt gag aag tgc ccc acc tgc cca gat gcc tgc 1922
 Ser Tyr Gly Asp Thr Cys Glu Lys Cys Pro Thr Cys Pro Asp Ala Cys
 620 625 630

acc ttt aag aaa gaa tgt gtg gag tgt aag aag ttt gac cgg gag ccc 1970
 Thr Phe Lys Lys Glu Cys Val Glu Cys Lys Lys Phe Asp Arg Glu Pro
 635 640 645 650

tac atg acc gaa aat acc tgc aac cgt tac tgc cgt gac gag att gag 2018
 Tyr Met Thr Glu Asn Thr Cys Asn Arg Tyr Cys Arg Asp Glu Ile Glu
 655 660 665

tca gtg aaa gag ctt aag gac act ggc aag gat gca gtg aat tgt acc 2066
 Ser Val Lys Glu Leu Lys Asp Thr Gly Lys Asp Ala Val Asn Cys Thr
 670 675 680

tat aag aat gag gat gac tgt gtc gtc aga ttc cag tac tat gaa gat 2114
 Tyr Lys Asn Glu Asp Asp Cys Val Val Arg Phe Gln Tyr Tyr Glu Asp
 685 690 695

tct agt gga aag tcc atc ctg tat gtg gta gaa gag cca gag tgt ccc 2162
 Ser Ser Gly Lys Ser Ile Leu Tyr Val Val Glu Glu Pro Glu Cys Pro
 700 705 710

aag ggc cct gac atc ctg gtg gtc ctg ctc tca gtg atg ggg gcc att 2210
 Lys Gly Pro Asp Ile Leu Val Val Leu Leu Ser Val Met Gly Ala Ile
 715 720 725 730

ctg ctc att ggc ctt gcc gcc ctg ctc atc tgg aaa ctc ctc atc acc 2258
 Leu Leu Ile Gly Leu Ala Ala Leu Leu Ile Trp Lys Leu Leu Ile Thr
 735 740 745

atc cac gac cga aaa gaa ttc gct aaa ttt gag gaa gaa cgc gcc aga 2306
 Ile His Asp Arg Lys Glu Phe Ala Lys Phe Glu Glu Glu Arg Ala Arg
 750 755 760

gca aaa tgg gac aca gcc aac aac cca ctg tat aaa gag gcc acg tct 2354
 Ala Lys Trp Asp Thr Ala Asn Asn Pro Leu Tyr Lys Glu Ala Thr Ser
 765 770 775

acc ttc acc aat atc acg tac cgg ggc act taa tgataagcag tcatcctcag 2407
 Thr Phe Thr Asn Ile Thr Tyr Arg Gly Thr
 780 785

atcattatca gcctgtgccca ggattgcagg agtccctgcc atcatgttta cagaggacag 2467

tattttgtggg gagggatttc ggggctcaga gtggggtagg ttggggagaat gtcagtatgt 2527

ggaagtgtgg gtctgtgtgt gtgtatgtgg gggctctgtgt gtttatgtgt gtgtgttgtg 2587

tgtgggagtg tgtaatttaa aattgtgatg tgcctgata agctgagctc cttagccttt 2647

gtcccagaat gcctcctgca gggattcttc ctgcttagct tgagggtgac tatggagctg 2707

agcaggtgtt cttcattacc tcagtgaaga gccagcttcc ctcacagggc cattgtccct 2767

gaagagaagg gcagggctga ggcctctcat tccagaggaa gggacaccaa gccttggtc 2827
taccctgagt tcataaattt atggttctca ggctgactc tcagcagcta tggtaggaac 2887
tgctggcttg gcagcccggg tcatctgtac ctctgectcc tttccctcc ctcaggccga 2947
aggaggagtc agggagagct gaactattag agctgctgt gccttttgcc atccctcaa 3007
cccagctatg gttctctcgc aagggaagtc cttgcaagct aattcttga cctgttgga 3067
gtgaggatgt ctgggccact caggggtcat tcatggcctg ggggatgtac cagcatctcc 3127
cagttcataa tcacaaccct tcagatttgc cttattggca gcg 3170

<210> 2
<211> 23
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 2
tttaccactg atgccaagac tca 23

<210> 3
<211> 21
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 3
ccgtcattag gctggacaat g 21

<210> 4
<211> 25
<212> DNA
<213> Artificial Sequence

<223> PCR Probe

<400> 4
atagcattgg acggaaggct ggcag 25

<210> 5
<211> 19
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 5
gaaggtgaag gtcggagtc 19

10/018445
581 Rec'd PCT/ 13 DEC 2001

<210> 6
<211> 20
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 6
gaagatggtg atgggatttc

20

<210> 7
<211> 20
<212> DNA
<213> Artificial Sequence

<223> PCR Probe

<400> 7
caagcttccc gttctcagcc

20

<210> 8
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 8
gcattctcgtc cgcctccc

18

<210> 9
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 9
gccagcacag tcacccag

18

<210> 10
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 10
actcactggg aactcgat

18

<210> 11
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 11

tggatggaga aattcttc

18

<210> 12

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 12

gttctggatg ctccacag

18

<210> 13

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 13

gccccgaagc caatccgc

18

<210> 14

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 14

aagcgggtca cctggtca

18

<210> 15

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 15

cattcctcca gccaatct

18

<210> 16

<211> 18

<212> DNA

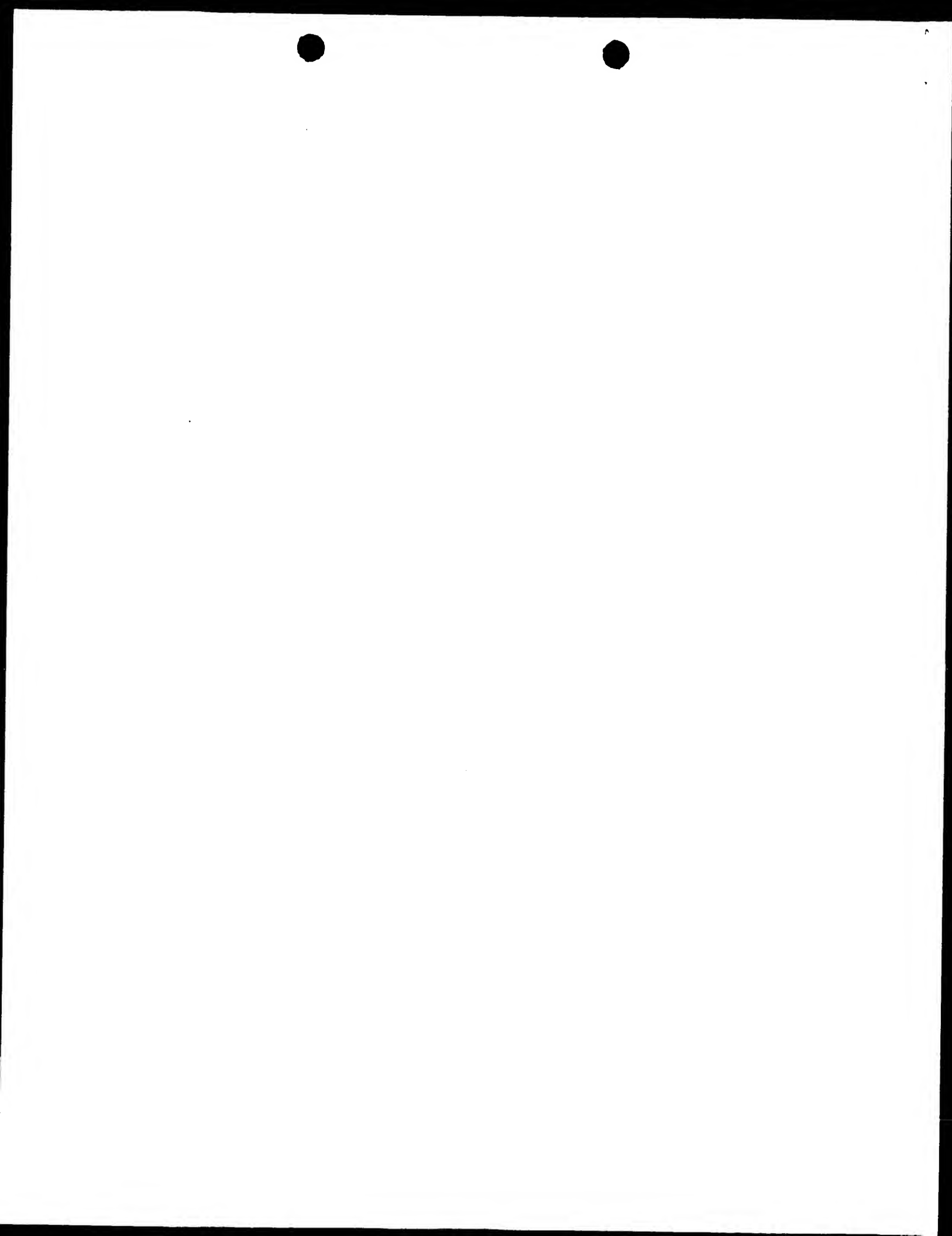
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 16

cttccgtcca atgctata

18



<210> 17
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 17
tgtcactacc aacatgac

18

<210> 18
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 18
agcttctcag tcatcagc

18

<210> 19
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 19
agagattgac tacatttt

18

<210> 20
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 20
ctttagaacg gatatttc

18

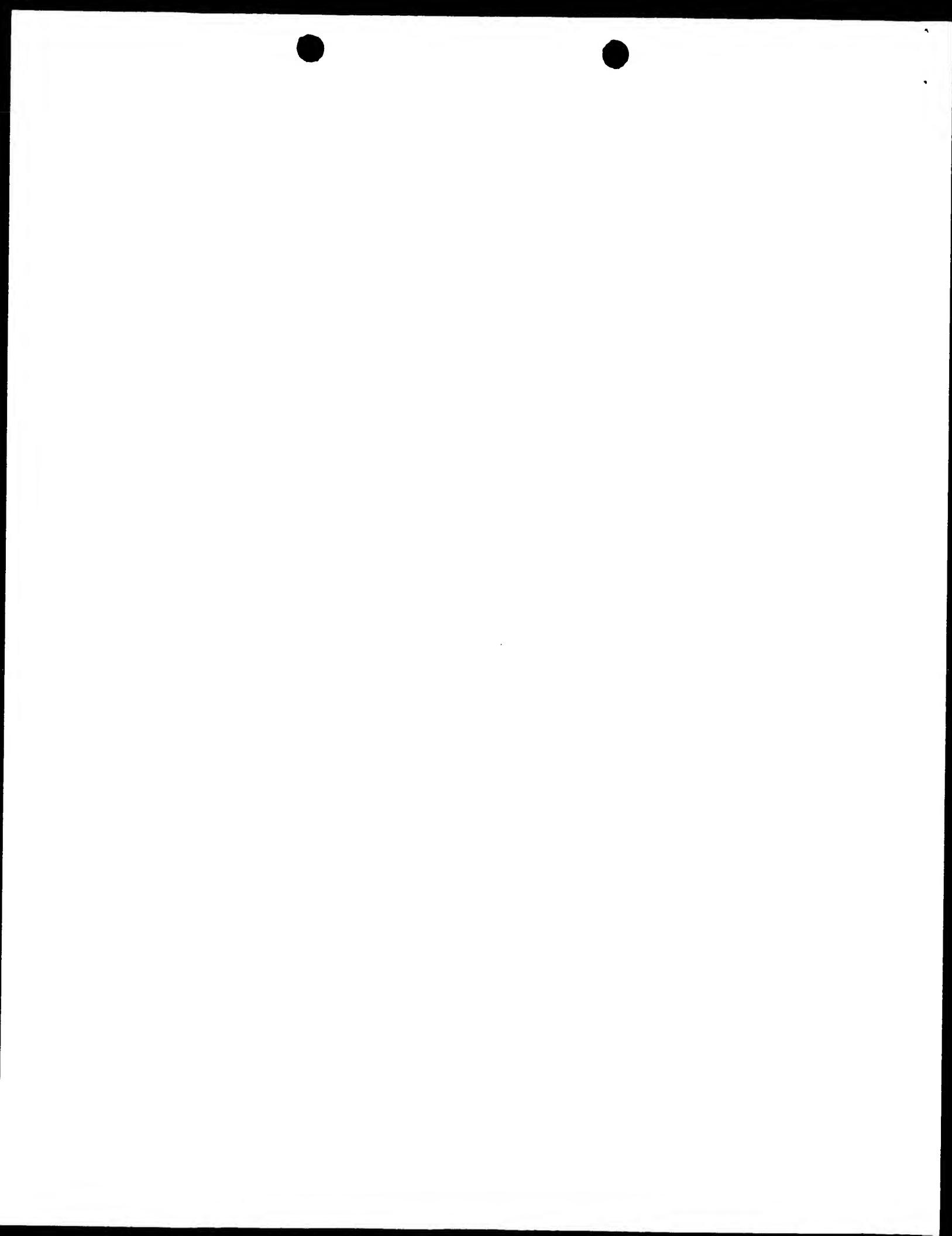
<210> 21
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 21
agacaactct tcagggag

18

<210> 22
<211> 18
<212> DNA



<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 22

tcaccgtgtc tccaatct

18

<210> 23

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 23

ccacgggctt tatggtaa

18

<210> 24

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 24

tcagcttggg cctggcag

18

<210> 25

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 25

agtcctcctc tgagcact

18

<210> 26

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 26

cacattgacc acagaggc

18

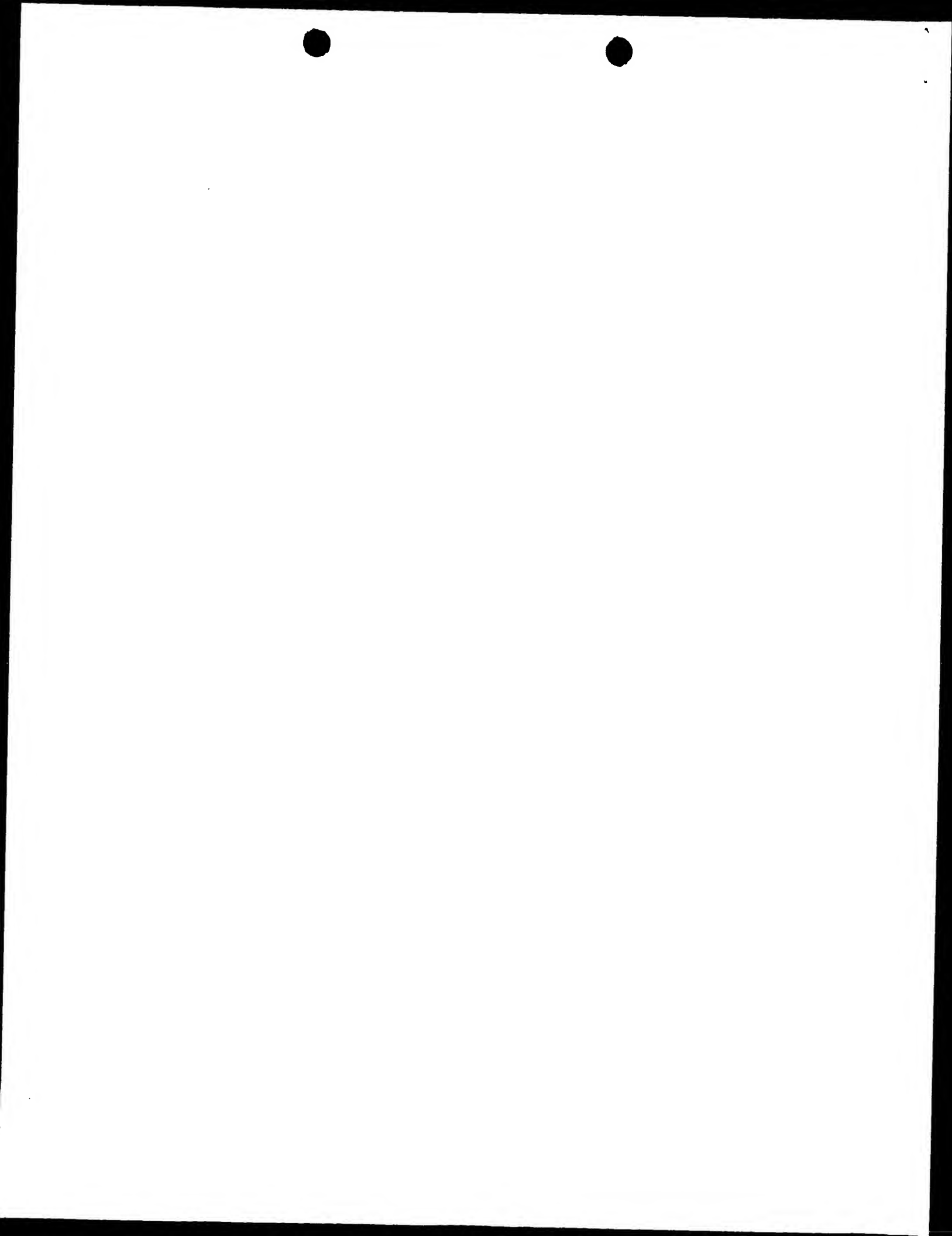
<210> 27

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide



<400> 27
agaagtcgtc acactcgc

18

<210> 28
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 28
gcccattgct ggacatgc

18

<210> 29
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 29
aggcatctgg gcaggtagg

18

<210> 30
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 30
tcaaacttct tacactcc

18

<210> 31
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 31
gcacccctgc cagtgtcc

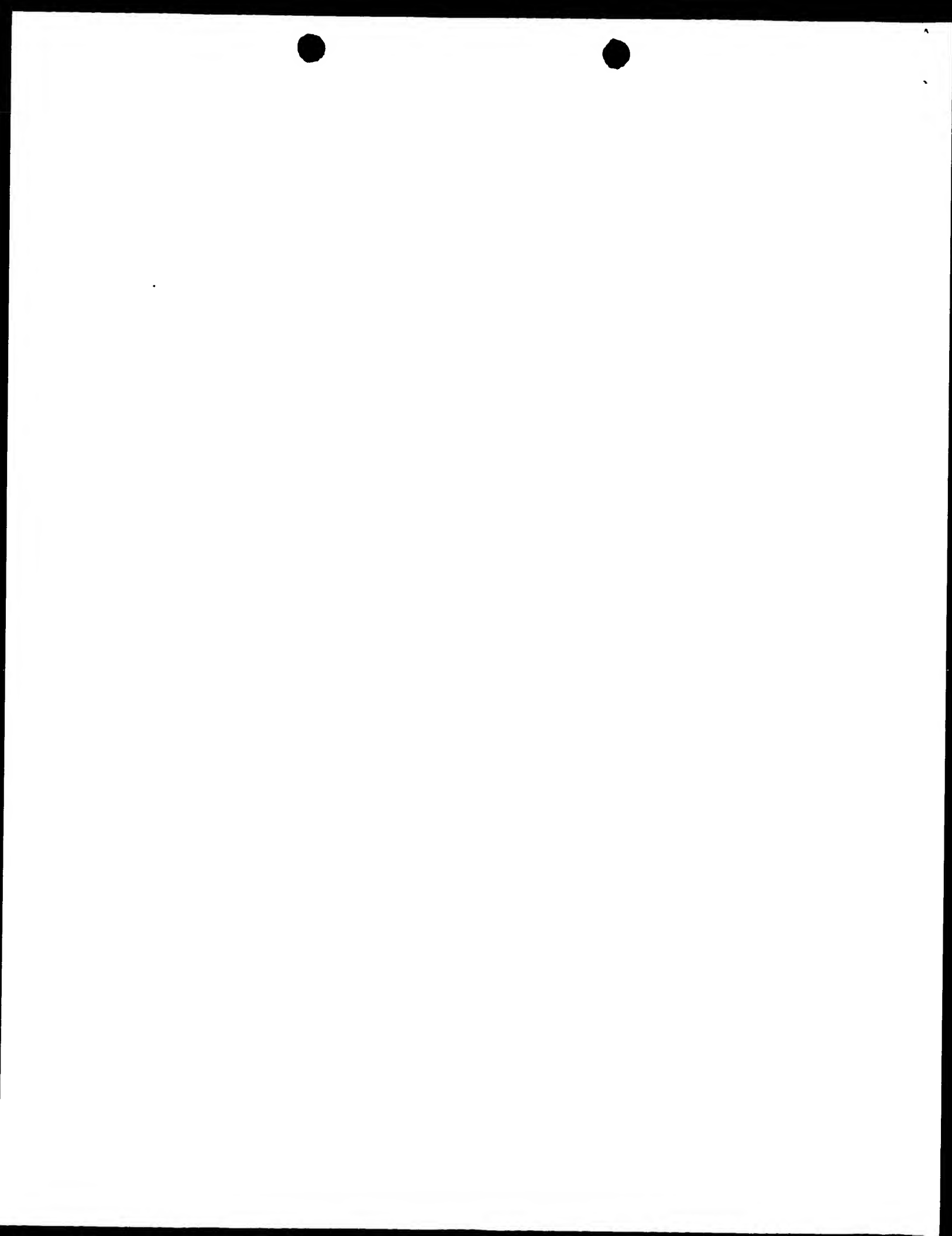
18

<210> 32
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 32
acgacacagt catcctca

18



<210> 33
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 33
accacatata ggatggac

18

<210> 34
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 34
gtgatgagga gtttccag

18

<210> 35
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 35
tgtcccatTT tgctctgg

18

<210> 36
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 36
aaggtagacg tggcctct

18

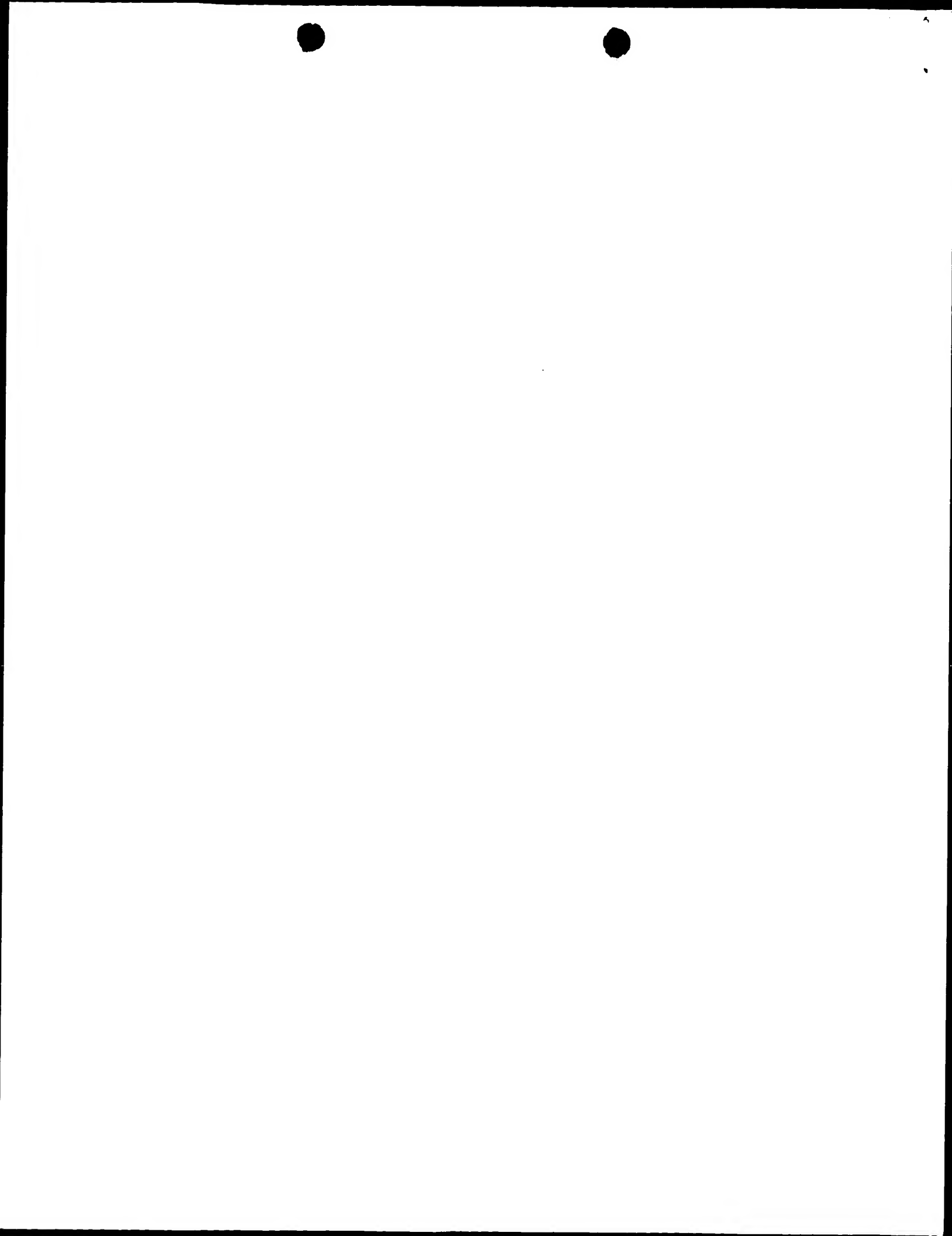
<210> 37
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 37
atgactgctt atcattaa

18

<210> 38
<211> 18
<212> DNA
<213> Artificial Sequence



<223> Antisense Oligonucleotide

<400> 38

gactcctgca atcctggc

18

<210> 39

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 39

acatactgac attctccc

18

<210> 40

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 40

acacacacat aaacacac

18

<210> 41

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 41

tcaccctcaa gctaagca

18

<210> 42

<211> 18

<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 42

tctcactgag gtaatgaa

18

<210> 43

<211> 18

<212> DNA

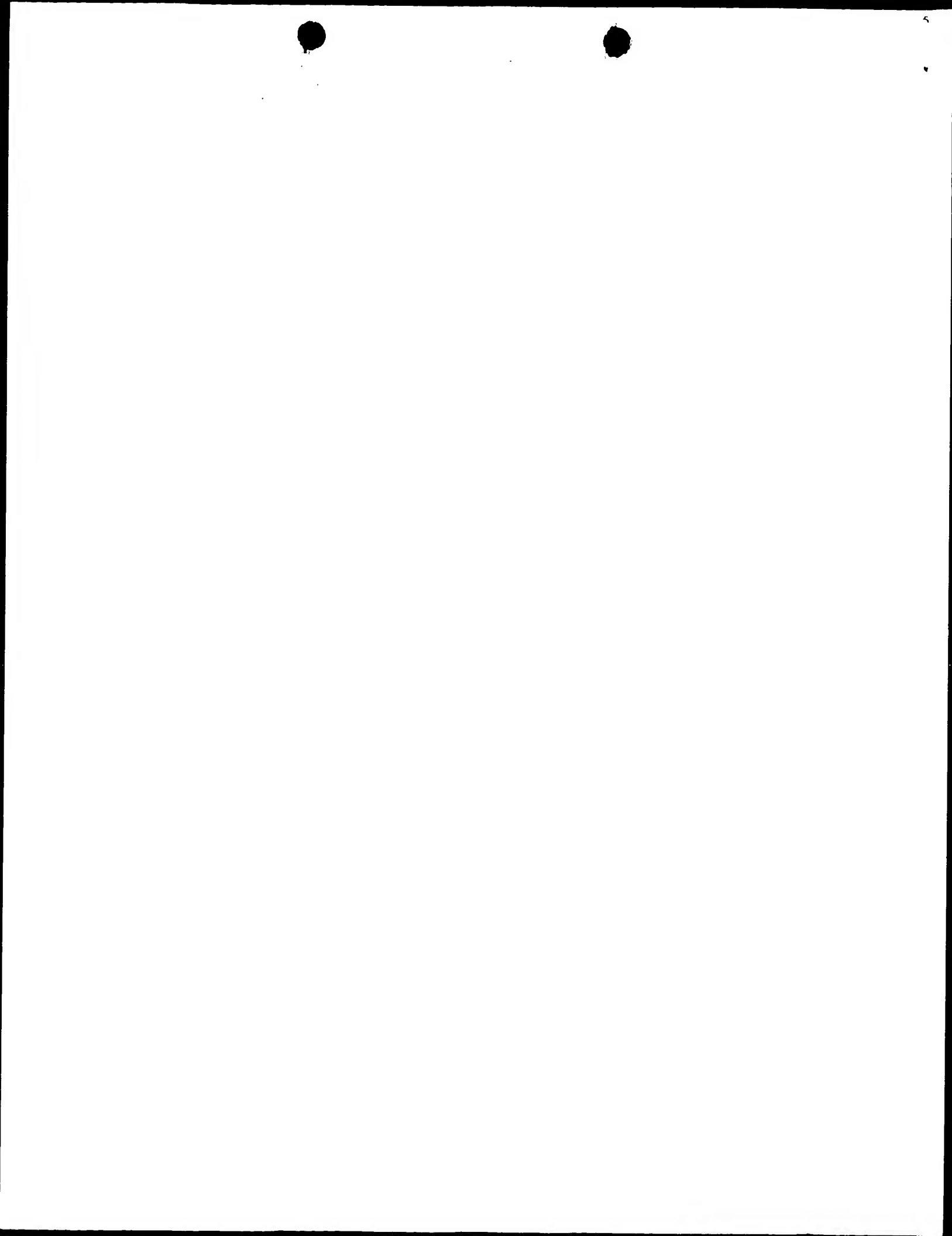
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 43

agctgctgag agtcaggc

18



<210> 44
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 44
tccttcggcc tgagggag 18

<210> 45
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 45
aaaggcacag gcagctct 18

<210> 46
<211> 18
<212> DNA
<213> Artificial Sequence

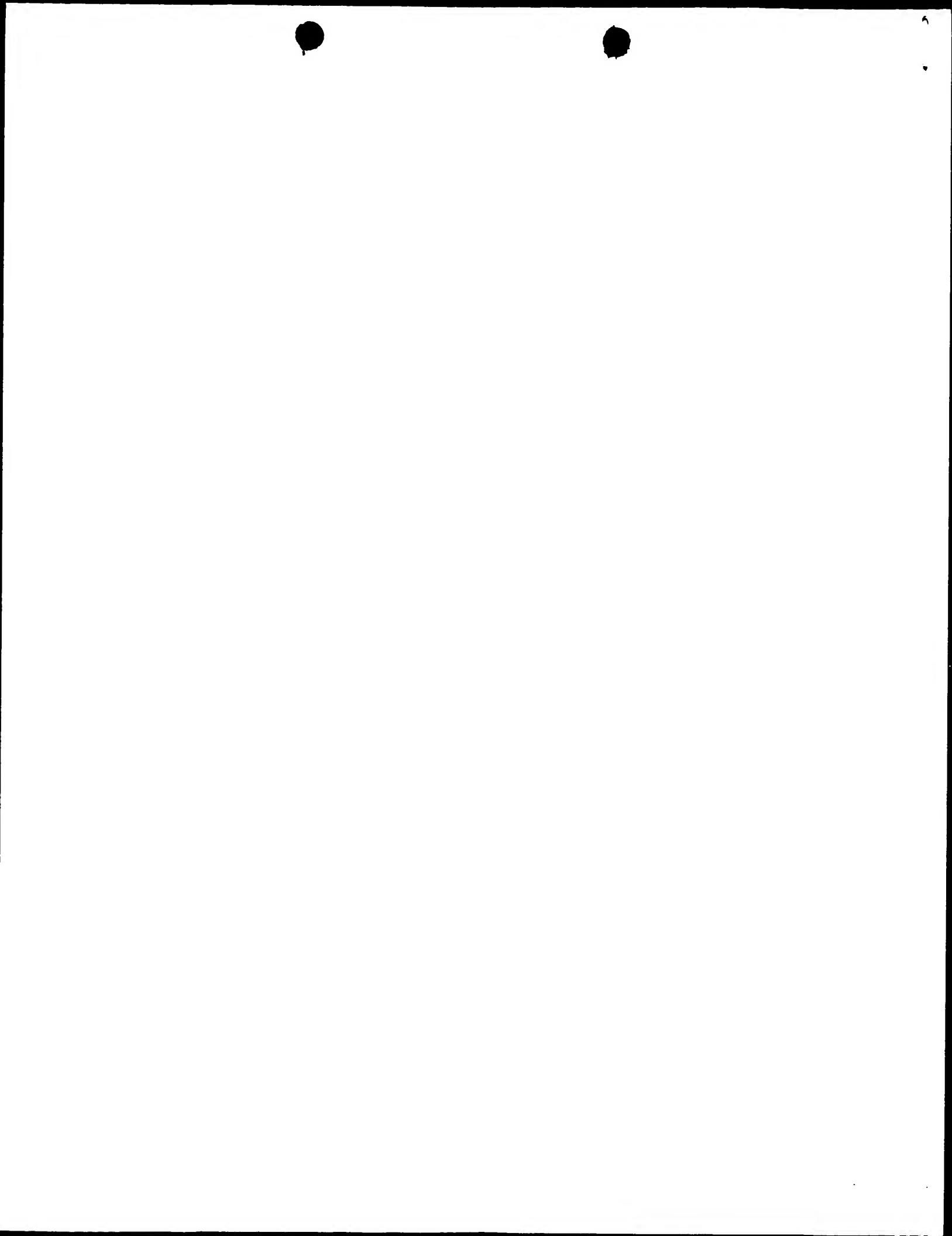
<223> Antisense Oligonucleotide

<400> 46
agatgctggt acatcccc 18

<210> 47
<211> 18
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 47
caataaggca aatctgaa 18



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/00633

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07H 21/02, 21/04; A61K 48/00; C12N 15/85; C12Q 1/68

US CL :536/23.1, 24.5; 435/6, 325, 366; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.5; 435/6, 325, 366; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, EMBASE, SCISEARCH, CAPLUS, BIOTECHNOBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	DALLABRIDA et al. Angiogenesis inhibition in vitro by integrin beta 3 antisense expression. FASEB J. 24 April 1998, Vol. 12, No. 5, page A1346, see panel 210.	1, 2, 12, 14, 15 ----- 3-11, 13
X - Y	DALLABRIDA et al. Inhibition of angiogenesis in vitro by integrin beta 3 antisense expression. Blood. 15 November 1997, Vol. 90, No. 10, Part 1, Supp. [1], page 286a, see panel 1261.	1, 2, 12, 14, 15 ----- 3-11, 13
Y	AKHTAR et al. Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). NAR. 1991, Vol. 19, No. 20, pages 5551-5559, see entire document.	3-11, 13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

07 MARCH 2000

Date of mailing of the international search report

31 MAR 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Andrew Wang

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/00633

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,789,573 A (BAKER et al) 04 August 1998, see entire document.	3-11, 13
Y	SANGHVI, Y.S. Heterocyclic base modifications in nucleic acids and their applications in antisense oligonucleotides. Antisense Research and Applications. 1993, pages 273-288, see entire document.	3-11, 13